

27.7.2015

MONDAY, 7/27

Petra, Tamanna, Tuukka

Made 1,2 % agarose gel with ETBR and run 5 ul sample of yesterday's Colony PCR results for Ycia-sfp-CAR part 1 (AH035) with 1 ul 6x LD. Used also Gene O'Ruler 1 kb ladder. Ran the gel 30 min with 120 V. The gel electrophoresis results can be seen in Fig 1 which samples 1-6 after the ladder well represents the colonies A8-A13 in an numerical order and sample 7 represents AH043 which contains the needed backbone pSB6A1. For each Colony PCR sample, the overnight culture at room temperature over the weekend was made in the Agar-plate containing kanamycin on friday. The plate is represented in Fig 2.

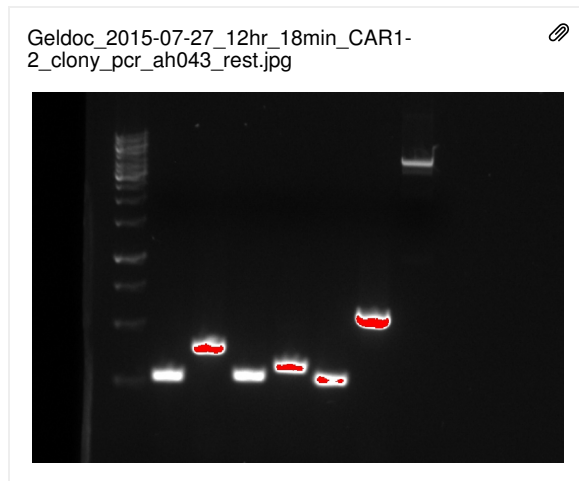


Fig 1. The gel electrophoresis results for colony PCR samples A8-A13. Contains also AH043 in the sample well 7.

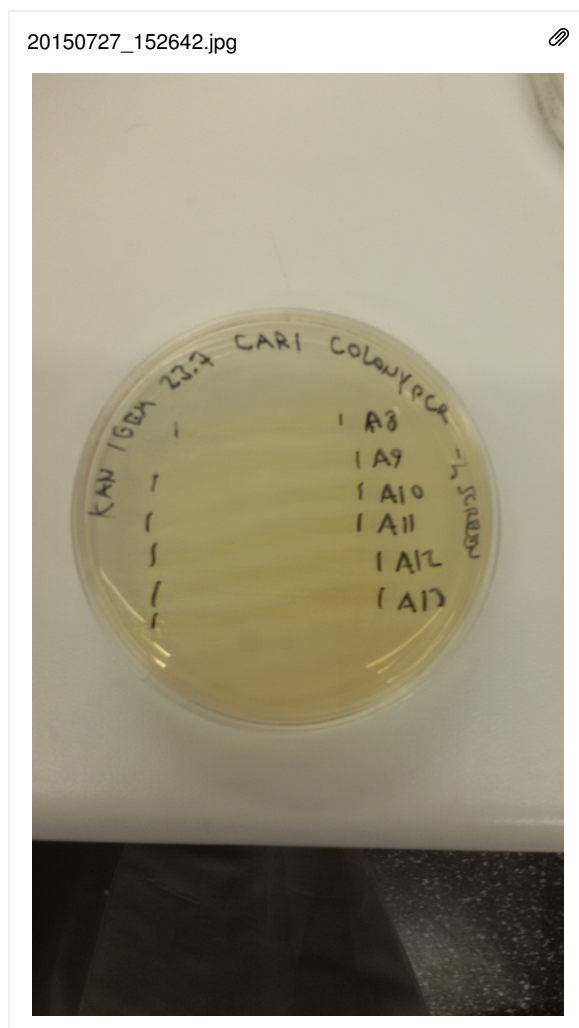


Fig 2. Screened colonies for PCR from the Blunt End Cloning.

The screening of colonies A8-A13 resulted too small sequences (1945 bp needed) so the new screening was done from the original plate of Blunt End Cloning (TOPO) transformation for Ycia-sfp-CAR part 1 (AH035). The colonies A14-A19 were picked up from the plate which is represented in Fig 3. Did the following reaction mix for each sample:

1,25 ul primers P0001 & P015
2 ul template DNA
8 ul water
12,5 ul 2x Mastermix
Total volume: 25 ul



Fig 3. The picked up colonies A14-A19 for the colony PCR.

Did colony PCR for CAR part 3, colony B6 (TOPO cloned & screened to contain right plasmid). Followed colony PCR protocol but did the PCR according to Ycia-sfp-CAR part 3 PCR protocol.

Reaction mix:
1,25 ul primers P011 & P018
2 ul template DNA
8 ul water
12,5 ul 2x Mastermix
Total volume: 25 ul

Run PCR following the protocol, but used 68,6 C annealing temperature.

Made 1,3 % agarose gel with ETBR and run 5 ul sample of the Ycia-sfp-CAR part 3 PCR reaction on gel with 1 ul LD. Used Gene O'Ruler 1 kb ladder. Ran the gel 25 min with 120 V. The gel electrophoresis results can be seen in Fig 4.

EDITED_CPCR_CAR_part_3_Geldoc_2015-07-27_15hr_50min.jpg



Fig 3. Gel electrophoresis image for Ycia-sfp-CAR part 3 PCR from the sample B6 (TOPO transformant)

It seems that PCR amplification failed. Possible failures may be resulted from wrong pipetting of the template DNA or primers.

We got our fixed Ycia-sfp-CAR part 2 GBlock (1000 ng) from IDT today. New CAR2 was ordered, because an illegal restriction site was found in the original one. Prepared the GBlock for use: made a 100 ng/ul stock.

- Centrifuged for 3 sec
- Added 10 ul sterilized water
- Vortexed briefly
- Incubated at 50 °C for 20 min
- Vortexed and centrifuged briefly

Made a 10 ng/ul stock of fixed CAR part 2

- 1 ul fixed CAR part 2 (100 ng/ul) to 9 ul sterilized water

PCR for fixed CAR part 2

Made a 0,9 ng/ul dilution of fixed CAR part 2

- 1 ul fixed CAR part 2 (10 ng/ul) stock to 10 ul sterilized water
- discarded the dilution after use

Prepared PCR reaction mix for fixed CAR part 2 according to protocol:

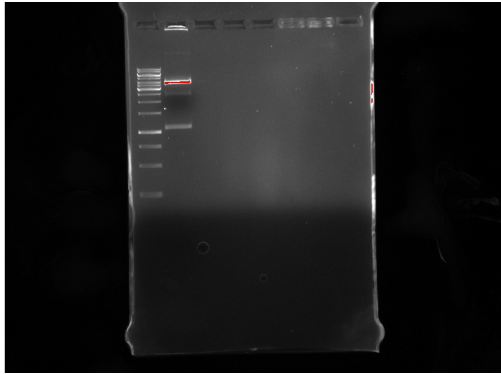
- + 1,1 ul fixed CAR part 2 dilution -> 1 ng template DNA
- + 8,9 ul water

Ran the PCR reaction according to protocol.

Did o/n culture of CAR part 3 colony B6 in LB with 2 µl KAN.

Made 1,3 % agarose gel with EtBr. Did restriction of AH043 (pSB6A1) following mostly the protocol. However, the incubation in 37 °C was 1,5 h long. Ran the restriction of AH043 in the gel for 45 min with 120 V.

Geldoc_2015-07-27_18hr_08min_AH043RFP.jpg



Did TOPO® Cloning reaction of AtoB part 1 (AtoB1) following mostly the protocol. However, the incubation in RT was 10 min. Then transformed AtoB1 to Top10 chemically competent cells following mostly the protocol. AtoB1 was plated 20 μ l & 50 μ l in KAN plates.

28.7.2015

TUESDAY, 7/28

Did gel purification of AH043 (pSB6A1) without insert following the GeneJET Gel Extraction kit protocol. Added 58,2 µl binding buffer.

NanoDrop result of AH043 without insert:

Table1

Sample	DNA (ng/ µl)	A260/A280
AH043 without insert	1,2	1,59

Did another colony PCR for Ycia-sfp-CAR part 3 (colony B6) since the one did yesterday didn't work.

Started screening AtoB part 1 TOPO colonies to find out which colonies contain the right plasmid. Chose 6 colonies (A1 1-6) for colony PCR.

Made new primer dilutions of primers P001, P005, P011 and P018.

- 1 ul primer to 9 ul sterilized water, final concentration 10 uM

Prepared template DNA for colony PCR's according to colony PCR protocol.

Reaction mixes for both of the reactions:

As in PCR protocols

+ 2 ul right template DNA

+ 8 ul sterilized water

Ran PCR reactions for Ycia-sfp-CAR part 3 and AtoB part 2 according to their PCR protocols.

Did a 1,2 % agarose gel with ETBR. Ran 5 ul samples with 1 ul LD of yesterday's and today's PCR reactions (14 samples in total). Used 2 ul Gene O'Ruler 1 kb ladder. Ran the gel for 25 minutes, 120 V.

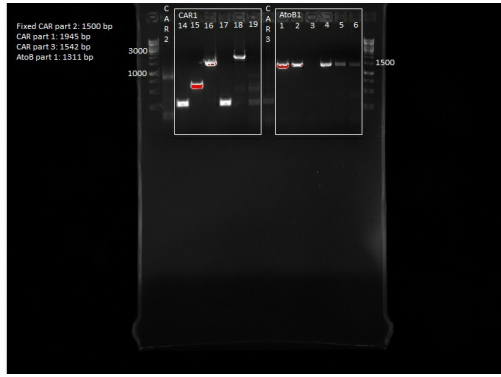
Pipetting order:

1. Ladder
2. Fixed CAR part 2
3. CAR part 1 colony PCR, colony 14
4. CAR part 1 colony PCR, colony 15
5. CAR part 1 colony PCR, colony 16
6. CAR part 1 colony PCR, colony 17
7. CAR part 1 colony PCR, colony 18
8. CAR part 1 colony PCR, colony 19
9. CAR part 3 colony PCR, colony B6
10. AtoB part 1 colony PCR, colony 1
11. AtoB part 1 colony PCR, colony 2
12. AtoB part 1 colony PCR, colony 3
13. AtoB part 1 colony PCR, colony 4
14. AtoB part 1 colony PCR, colony 5
15. AtoB part 1 colony PCR, colony 6
16. Ladder

All the colony PCR's were made from TOPO cloned colonies. Only CAR part 3, colony B6 is known to contain the right plasmid.

Gel results:

EDITED_fCAR2_CAR1_CAR3_AtoB1_Geldoc_2015-07-28_15hr_00min.jpg



According to gel picture right-sized plasmids were found from the following reactions:

7. CAR part 1 colony PCR, colony 18
10. AtoB part 1 colony PCR, colony 1
11. AtoB part 1 colony PCR, colony 2
13. AtoB part 1 colony PCR, colony 4
14. AtoB part 1 colony PCR, colony 5
15. AtoB part 1 colony PCR, colony 6

The colony samples A14-A19 for Ycia-sfp-CAR part 1 TOPO strain were also screened with o/n plate which positions can be seen in Fig 2. From the gel sample, the A18 had the right size of DNA (1945 bp).

20150728_173704.jpg

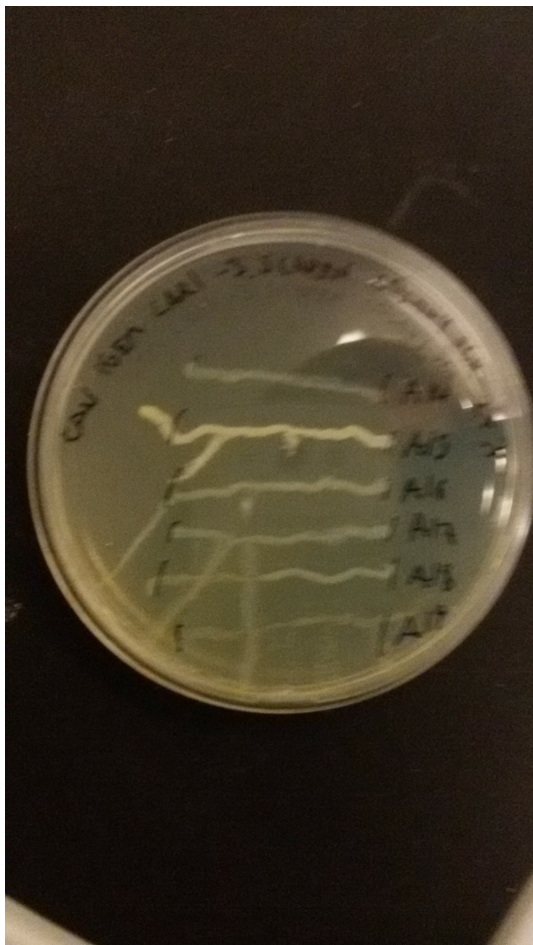


Fig 2. Screened colonies A14-A19 (Ycia-sfp-CAR part 1) for the colony PCR

We still need to figure out why PCR reaction for fixed CAR part 2 and Colony PCR for CAR part 3, colony B6 doesn't work.

Did PCR reaction of CenA+Cex part 1 (CenA1) and CenA+Cex part 2 (CenA2) following their respective protocol. For the reactions diluted for the first time primers p002, p003 and p004 and synthesized DNA fragments CenA1 and CenA2. For CenA1 followed the GeneScript protocol to do dilution. For CenA2, first spinned in RT and 6000 rcf for 1 min and then incubated for 1 h with 50 μ l sterile water. In the end, the concentration of CenA1 was 200 ng/ μ l and of CenA2 was 100 ng/ μ l. For PCR reaction DNA fragments were further diluted in different tubes with sterile water.

Did miniprep of CAR3 colony B6 from the o/n culture following the kit protocol.

NanoDrop result of CAR3 B6:

Table2

Sample	DNA (ng/ μ l)	A260/A280
CAR3 B6	235,1	1,85

Did PCR reaction of CAR3 B6 and colony PCR reaction of CAR1 A6 to check if there haven't been any mishaps. Followed their respective protocols to do the reactions.

Made heat shock transformation for the commercial plasmid pCDFDuet1 which arrived today from the shipping. Two streptomycin agar plates (50 μ l and 100 μ l) were labeled with the transformants for the overnight cultivation (18h, 37C and 230 RPM).

29.7.2015

WEDNESDAY, 7/29

Petra, Tamannae, Tuukka

Checked transformation plates (50ul and 100 ul) for pCDFDuet-1. The surface was almost fully covered with the colonies, so did the overnight culture (24h) with 2ml LB which contained 50 ug/ml streptomycin.

Gradient KAPA hot start PCR for fixed Ycia-sfp-CAR part 2

Tested KAPA hot start PCR kit for the first time. To find out the right annealing temperature we did a gradient PCR. For gradient PCR 13 x reaction mix was made and divided into 12 tubes.

13x reaction mix (325 ul):

217,75 ul sterilized water

65 ul 5x buffer

9,75 ul 10mM dNTP mix

9,75 ul 10μM P016

9,75 ul 10μM P017

6,5 ul KAPA HiFi HotStart DNA Polymerase

6,5 ul fixed CAR part 2 DNA dilution (1 ng/ul)

Gradient PCR protocol for fixed Sfp-ycia-CAR part 2:

95° C - 3 min

98° C - 20 sec

55-69° C - 15 sec

72° C - 60 sec

72° C - 1,5 min

4° C - forever

Repeated underlined cycles 25 times

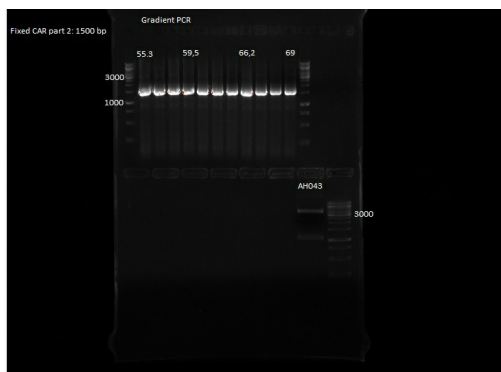
Made an 1,3 % agarose gel with ETBR.

Upper row: Ran in total 11 5 ul samples of fixed Sfp-ycia-CAR part 2 gradient PCR reaction with 1 ul LD on gel to check the results.

Lower row: Ran 25 ul restricted AH043 backbone with 5 ul LD to clean it out of the gel.

Used GeneO'Ruler 1 kb ladder.

EDITED_GPCR_FCAR2__AH043_Geldoc_2015-07-29_15hr_07min.jpg



Gel results

PCR purifications: all done with GeneJET PCR purification kit according to the kit protocol.

Purified the last 4 PCR reactions. Put all the reactions to the same purification column and used 30 ul elution buffer.

Purified CAR part 1, colony 18 from yesterday. Used 25 ul elution buffer.

Purified AtoB part 1, colonies 1,2,4,5 and 6 frm yesterday. Mixed all to the same purification column. Used 30 ul elution buffer.


Nanodrop results:

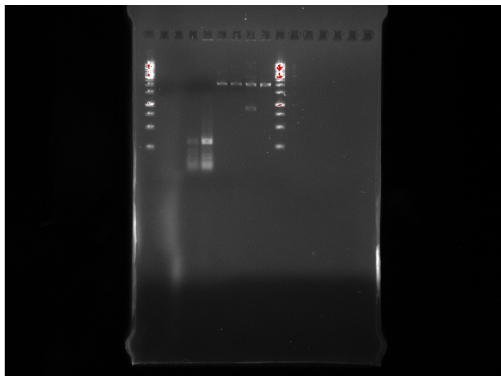
Table1

Construct	Concentration (ng/ul)	A260/A280
Ycia-sfp-CAR part 1	53,7	1,84
Fixed Ycia-sfp-CAR part 2	224,7	1,86
AtoB part 1	106,1	1,85

Did restriction of AH043 (pSB6A1) again following mostly the protocol. However, the incubation in 37 °C was 1,5 h long.

Made 1,3 % agarose gel with EtBr. Run yesterday's PCR product of Cen+Cex part 1 (CenA1) and Cen+Cex part 2 (CenA2) and colony PCR products of CAR3 B6 and CAR1 A6 on the gel for 45 min with 120 V. Pipeting order was 1. ladder 2 µl 2. CenA1 6 µl 3. CenA2 6 µl 4. CAR3 B6 6 µl 5. CAR1 A6 6 µl.

Geldoc_2015-07-29_12hr_01min_Cen_ceX_ahd1_ahd3_ahd4_ahd5.jpg 



Results for CenA1 and CenA2 were that nothing were amplified and for the colony PCR reactions were that colonies were mixed so we don't have correct CAR3. That's why we decided that we'll try to do PCR reactions again with KAPA.

Made KAPA PCR mix for CenA1 without template DNA and HotStart DNA Polymerase and stored in -20 °C (more info about mix components will be in the next day's lab note).

30.7.2015

THURSDAY, 7/30

Petra, Tamannae, Tuukka

Made the NucleoSpin Plasmid EasyPure miniprep for the transformants of pCDFDuet-1 to obtain 50ul of eluent/DNA. The following Nanodrop results were gathered:

Nanodrop results:

Table1

Construct	Concentration (ng/ul)	A260/A280
pCDFDuet-1	7,0	1,84

Gradient KAPA hot start PCR for fixed Ycia-sfp-CAR part 3, AtoB part 3 and AtoB part 4.

To find out the right annealing temperature we did a gradient PCR. For gradient PCR 13 x reaction mix was made and divided into 12 tubes.

Made DNA template dilutions

- 1 ul DNA (10 ng/ul) to 9 ul water

Made primer dilutions

- 1 ul primer (100 ng/ul to 9 ul water)

13x reaction mix (325 ul):

217,75 ul sterilized water

65 ul 5x buffer

9,75 ul 10mM dNTP mix

9,75 ul 10μM reverse primer

9,75 ul 10μM forward primer

6,5 ul KAPA HiFi HotStart DNA Polymerase

6,5 ul fixed CAR part 2 DNA dilution (1 ng/ul)

Sfp-ycia-CAR part 3: 1542 bp, P018 and P011

AtoB part 3: 1772 bp, P008 and P009

AtoB part 4: 1414 bp, P010 and P011

Gradient PCR protocol for fixed Sfp-ycia-CAR part 3, AtoB part 3 and AtoB part 4:

95° C - 3 min

98° C - 20 sec

58-72° C - 15 sec

72° C - 70 sec

72° C - 100 sec

4° C - forever

Repeated underlined cycles 25 times

Gradient:

58,0 °C

58,3 °C

59,3 °C

60,8 °C

62,5 °C

64,2 °C
65,8 °C
67,5 °C
69,2 °C
70,7 °C
71,7 °C
72,0 °C

Made two 1,2 % agarose gels with ETBR. Added 2 thin combs to the first gel and one to the second gel. Ran 5 ul samples with 1 ul ladder of each PCR reaction on gel (36 samples in total).

Pipetting order:

Gel1, upper row:

CAR part 3

Ladder 1 2 3 4 5 6 **8** 7 9 10 11 12 Ladder

Gel1, lower row:

AtoB part 3

Ladder 1 2 3 4 6 **5** 7 8 9 10 11 12 Ladder

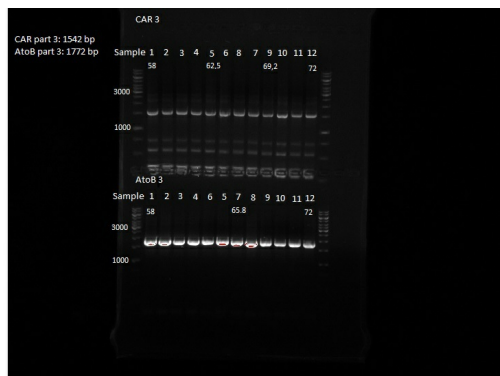
Gel2

AtoB part 4

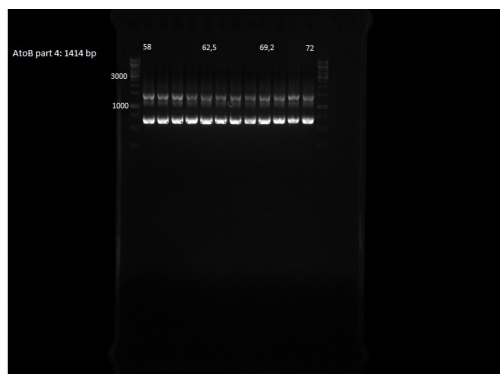
Ladder 1 2 3 4 5 6 7 8 9 10 11 12 Ladder

Results:

EDITED_GPCR_CAR3_ATOB3_Geldoc_2015-07-30_16hr_33min.jpg



EDITED_GPCR_AtoB4_Geldoc_2015-07-30_16hr_40min.jpg



Run the yesterday's restriction of AH043 in a 1,3 % agarose gel for 49 min with 120 V. Then did gel purification of AH043 following the kit protocol. Added 41, 4 µl binding buffer.

NanoDrop result of AH043:

Sample	DNA (ng/ul)	A260/A280
AH043	3,3	1,66

Did KAPA PCR reactions of Cen+Cex part 1 (CenA1) and Cen+Cex part 2 (CenA2). Added template DNA (CenA1) and DNA polymerase to the yesterday's CenA1 PCR mix. Below is the mixes for the both:

CenA1 13x reaction mix (325 ul):

217,75 ul sterilized water

65 ul 5x buffer

9,75 ul 10mM dNTP mix

9,75 ul 10μM P001

9,75 ul 10μM P003

6,5 ul KAPA HiFi HotStart DNA Polymerase

6,5 ul CenA1 DNA dilution (1 ng/ul)

CenA2 13x reaction mix (325 ul):

217,75 ul sterilized water

65 ul 5x buffer

9,75 ul 10mM dNTP mix

9,75 ul 10μM P016

9,75 ul 10μM P017

6,5 ul KAPA HiFi HotStart DNA Polymerase

6,5 ul fixed CAR part 2 DNA dilution (1 ng/ul)

The PCR program for CenA1 and CenA2 were similar only differing from gradient annealing temperatures which is shown in the pictures below.

The PCR program:

95°C - 3 min

98°C - 20 sec

56,5°C-71,5°C -15 sec

72°C - 75 sec

72°C - 1,5 min

4°C - forever

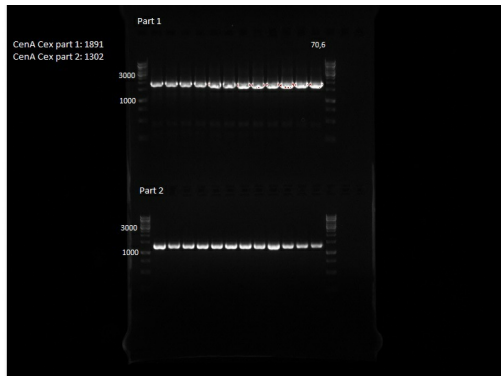
Repeated underlined cycles 25 times

Did 1,3 % agarose gel with EtBr with double layer. Ran PCR reactions of CenA1 and CenA2 for 25 min with 120 V. Pipeting order was:

I 1. ladder 2 μl 2.-13. CenA1 gradient 6 μl 14. ladder 2 μl

II 1. ladder 2 μl 2.-13. CenA2 gradient 6 μl 14. ladder 2 μl

EDITED_GPCR_CenA1CenA2_Geldoc_2015-07-30_14hr_21min_.jpg



So, the KAPA PCR reactions of CenA1 and CenA2 were successful.

Did gel purification of CenA1 following the kit protocol. First, the correct bands of CenA1 were cut to a tube. Added 534 μ l binding buffer. Rest of the PCR products were stored in the fridge.

NanoDrop result of CenA1:

Table3

Sample	DNA (ng/ μ l)	A260/A280
CenA1	122,0	1,90

Did PCR purification of CenA2 using PCR products of 9.-12. tubes following the kit protocol. Added 74 μ l binding buffer.

NanoDrop result of CenA2:

Table4

Sample	DNA (ng/ μ l)	A260/A280
CenA2	114,3	1,85

Did o/n culture of pACYC in LB with 2 μ l CAM and a new o/n culture of pCDF in LB with Strep.

31.7.2015

FRIDAY, 7/31

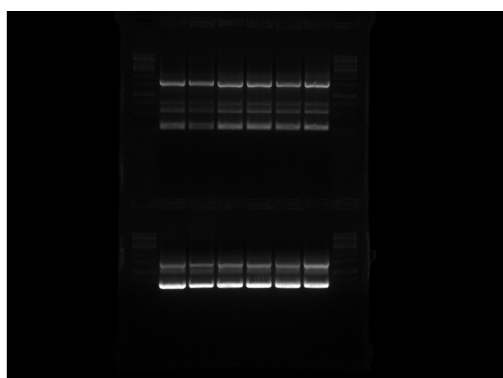
Petra, Tamannae

Made 1,3 % gel with ETBR. Ran yesterday's gradient PCR reactions (AtoB part 4 and Ycia-Sfp-CAR part 3). Ran 6 samples x 20 ul + 4 ul LD of both reactions :

- AtoB part 4: samples 6-11
- Ycia-Sfp-CAR part 3: samples 7-12

Ran the gel 120V, 25 min. Took a picture and continued the run for additional 8 minutes before cutting the correct DNA bands from gel.

Geldoc_2015-07-31_15hr_27min_CAR3_AtoB_extraction_1st_picture.jpg



Purified AtoB part 4 and Ycia-Sfp-CAR part 3 from gel using GeneJET gel extraction kit following the protocol. According to the gel weight added 670 ul binding buffer to CAR3 and 580 ul to AtoB4. Nanodrop results are in Table 1.

Purified AtoB part 3 from yesterday's gradient PCR reaction with GeneJET PCR purification kit according to the protocol. Did run the PCR reactions for 25 min with 120 V in a 1,3 % agarose gel. Purified samples 7-12. Nanodrop results are in Table 1.

Table1

Construct	Concentration (ng/ul)	A260/280
AtoB part 3	284,3	1,80
AtoB part 4	52,1	1,87
Ycia-Sfp-CAR part 3	70,8	1,83

Did minipreps of yesterday's o/n cultures of pACYC and pCDF Duet-1 following the kit protocol.

NanoDrop result of the minipreps:

Sample	DNA (ng/ul)	A260/A280
pACYC	28,2	1,96
pCDF Duet-1	13,6	1,97

Did KAPA PCR reactions of 2nd BglX and 2nd VioA. With these PCR reactions, we hope that suffixes will be added to already PCR amplified BglX and VioA parts.

2nd BglX 13x reaction mix (325 ul):

217,75 ul sterilized water

65 ul 5x buffer

9,75 ul 10mM dNTP mix

9,75 ul 10µM P011

9,75 ul 10µM P033

6,5 ul KAPA HiFi HotStart DNA Polymerase

6,5 ng BglX PCR product (done in 7.7.)

2nd VioA 13x reaction mix (325 ul):

217,75 ul sterilized water

65 ul 5x buffer

9,75 ul 10mM dNTP mix

9,75 ul 10µM P028

9,75 ul 10µM P029

6,5 ul KAPA HiFi HotStart DNA Polymerase

6,5 ng VioA PCR product

The PCR program for 2nd BglX and 2nd VioA were same.

The PCR program:

95°C - 3 min

98°C - 20 sec

54 C - 69 C -15 sec

72°C - 82 sec

72°C - 1,5 min

4°C - forever

Repeated underlined cycles 25 times

Stored PCR products in the fridge.